Role of the C355 – C385 Disulfide Bonds in PACE4 Function

By

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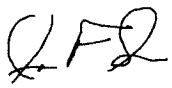

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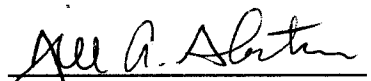

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Abstract

Furin and PACE4 are part of a family of proteins called the proprotein convertases. Both proteins are critical for development, homeostasis, and disease pathology. Both proteins have two disulfide bonds within the catalytic domain. In furin, the bonds are C211-C260 and C303-C333, while the corresponding bonds in PACE4 are C263-C412 and C355-C385. PACE4 is like furin in structure and function, which will allow us to investigate any similarities that exist between the trafficking and function of these two proteins. Site-directed mutagenesis was performed to change the individual cysteine residues to serine residues. PACE4 constructs and a pro-vWF construct were used to perform a co-expression assay to examine if the disulfide bond knockout abolished the ability of PACE4 to process pro-vWF. RPE.40 cells were used, because they are furin null and do no produce PACE4 protein. Additionally, an activity assay was performed to further examine the effects of removing a disulfide bond from PACE4. Only the C355S PACE4 mutant was generated. Co-expression assays showed that the C355-C385 disulfide bond was critical for the ability of PACE4 to process substrates. This study allowed us to gain information about the disulfide bonds and PACE4 function, which will allow us to corroborate our findings about furin maturation.

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Introduction

Proprotein convertases (PCs) are a group of calcium-dependent serine proteases that are responsible for the activation, and sometimes inactivation, of a wide variety of proteins. These proproteins are activated by PCs through the cleavage at specific basic and non-basic residues (Seidah, 2011). PCs are a family of nine different proteins: PC1/3, PC2, furin, PC4, PC5/6, paired basic amino acid cleaving enzyme 4 (PACE4), PC7, subtilisin kexin isozyme 1 (SKI-1), and proprotein convertase subtilisin/kexin type 9 (PCSK9). Two of the most important PCs are furin and PACE4 due to their involvement in diseases such as arthritis and cancer. These 2 proteins take part in many biological pathways by activating or inactivating hormones, growth factors, adhesion molecules, and viral and bacterial proteins. The cleavage carried out by furin and PACE4 occurs after a single or paired basic residue and within the same motif: Arg-X-X-Arg. Furthermore, PACE4 and furin cleave substrates at similar sequences, which equates to redundancy in the substrates they cleave.

Activation of furin is completed in the Golgi network. Furin can begin cleaving some of its substrates there, but it can also act at the cell surface or even in recycling endosomes. PACE4, on the other hand, is a secreted enzyme and usually cleaves its substrates in the extracellular environment, including at the cell surface. Therefore, the pool of substrates that is cleavable by furin is likely much larger compared to that of PACE4. Currently, the only substrates known to be unique to PACE4 are a disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4 and 5) (Nissinen and Kahari, 2012). Both of these matrix metalloproteases are directly involved

in osteoarthritis, while ADAMTS-5 is involved in malignant tumor progression (Nissinen and Kahari, 2012).

During embryological development of vertebrates, axis formation into specific anteroposterior and left-right axes is critical. Disruptions in the formation of these axes leads to birth defects and, in most instances, death. Molecules related to Transforming Growth Factor-Beta (TGF-B), such as Nodal, Lefty, and Bone Morphogenetic Proteins (BMPs), dictate the correct formation of these axes during development (Meno et al., 1996, 1997). These molecules are therefore critical and are subject to numerous regulatory mechanisms. One of these involves the endoproteolytic cleavage of inactive precursors of Nodal, Lefty, and BMPs. Currently, it is thought that this cleavage occurs in the *trans*-Golgi network before the proteins are secreted (Sha et al., 1989). Certain PCs, such as furin and PACE4, have been found to carry out the cleavage of these precursors (Cui et al., 1998). Currently, it is thought that PACE4 plays an important role in the maturation of Nodal (Constam and Robertson, 1999). Studies that introduced a non-functional PACE4 protein into mouse embryos showed that most embryos failed to develop complex craniofacial formations (Constam and Robertson, 2000). Additionally, PACE4 expression in the foregut is necessary for formation of the anterior central nervous system (Constam and Robertson, 2000). Mouse embryos that lacked functional furin protein failed to form several ventral closure points in addition to the formation of morphogenic heart defects (Zhang and Bradley, 1996). It is currently thought that furin activity is required for BMPs to be effectively processed and matured (Winnier et al., 1995; Zhang and Bradley, 1996).

In addition to embryological development, PACE4 and furin are involved in several disease pathologies. Both proteins help regulate key proteins in pathways associated with cancer. Significant pathways that are thought to be involved in cancer include the protein products of the *KRAS, RB1, CDKN2, TP53, SMAD4, and TGF-β1* genes. Other suspected pathways that are being examined include Hedgehog, NOTCH 1, AKT1-PI3K-MTOR, BRCA2-PALB2, and Fanconi pathways (Vincent et.al, 2011). PACE4 and furin are thought to be involved in the activation of several of these pathways. The TGF-β1 precursor is cleaved and activated by furin (Blanchette et al., 1997). NOTCH 1 is an epidermal growth factor that has been found to be involved in pancreatic cancer. Furin processes and activates full length NOTCH 1 (Ma et al., 2012). A recent study showed that PACE4 siRNA had antitumor activity in human pancreatic cancer that was achieved by inducing apoptosis through the mitochondrial signaling pathway (Tian et al., 2016).

In addition to their involvement in cancer, PACE4 and furin are also involved in arthritis. PACE4 is thought to be involved in cartilage degradation that is associated with osteoarthritis (OA). Recently, a clinical study linked specific single nucleotide polymorphisms in the *PCSK6* gene, which encodes PACE4, with pain perception in OA patients (Malfait et al., 2011). This suggests that PACE4 is involved in OA. Furthermore, PACE4 has been shown to cleave and activate ADAMTS-4 and ADAMTS-5 (Longpré et al., 2008). These are matrix-degrading proteinases that are responsible for the degradation of aggrecan, a key component of articular cartilage that is responsible for the resistance of compressive forces. Lack of aggrecan leads to increased joint damage. Proteoglycan levels, including aggrecan, are directly related to OA severity.

Lower levels have been found in more severe cases (Lahm et al., 2010). It is currently thought that furin is involved in the severity of rheumatoid arthritis (RA) and OA. Furin plays a role in the regulation of the immune system. Removal of furin in mouse T cells led to aberrant T-helper cell polarization (Pesu et al., 2008). Furthermore, furin plays a role in inhibiting the inflammation process in myeloid cells through the reduction in the amount of proinflammatory cytokines that are produced (Cordova et al., 2016). This suggests that furin plays a protective role for joints against RA (Wu et al., 2017). Furin is also thought to play a limiting role against OA by inhibiting matrix metalloproteinase-13 (Lin et al., 2018).

Until recently, the intracellular trafficking pathway for furin was thought to be completely understood. However, results in our lab show controversial details pertaining to furin trafficking and function. When synthesized, furin is produced as a zymogen that contains a signal peptide that is 24 amino acids in length and a propeptide that is 83 residues in length. Propeptide cleavage occurs autocatalytically in the endoplasmic reticulum (ER) on the carboxy-terminal end of NH2-R104-T105-K106-R107-COOH (Leduc et al., 1992). The propeptide is needed in furin activation and maturation for two reasons. First, the propeptide acts as an intramolecular chaperone and is necessary for the correct folding of furin. Second, the excised propeptide remains attached to furin via noncovalent interactions. The association of the propeptide with furin allows for autoinhibition, which is lifted when a second intramolecular cleavage occurs in the trans Golgi network (TGN) on the C-terminal side of NH2-R70-A71-V72-T73-K74-R75-COOH (Anderson et al., 2002). The propeptide dissociates from furin because of the cleavage, and furin is activated.

Furin trafficking is associated with two folding events that occur in different compartments. The first folding event occurs in the ER, which allows for furin to be transported to the endoplasmic reticulum Golgi intermediate compartment (ERGIC). The second folding event occurs in the ERGIC, allowing furin to be transported to the Golgi. Furthermore, localization to the TGN is mediated by the 23-residue transmembrane domain and certain sequences located in the cytoplasmic tail. The tail is recognized by proteins in Golgi, which cycle furin between the TGN and post-TGN vesicles (Thomas, 2002). There are also key sequences in the tail that are important for trafficking: the tyrosine-based motif Y_{760}KGL and the acidic cluster E_{769}ECPSDSEEDE$_{779}$. The tyrosine-based motif is linked to plasma membrane internalization and TGN sorting. The acidic cluster is necessary for TGN localization. S773 and S775 in the acidic cluster are phosphorylated by casein kinase II and dephosphorylated by protein phosphatase 2A (Thomas, 2002). These events have led to a two-loop trafficking model for furin (Thomas, 2002). The first loop is between the cell surface and sorting endosomes. The second loop consists of movement between the TGN and a late endosomal compartment. Included in this model is a clathrin-dependent step, where cell surface furin is endocytosed by interactions between AP-2 adaptor and an internalized tyrosine motif (Teuchert et al., 1999).

PACE4 is a protein that is broken down into 5 distinct domains that range from the N to C terminus, respectively: signal peptide, propeptide, catalytic domain, P domain, and the cysteine rich domain (Seidah, 2011). The trafficking pathway of PACE4 is similar to furin. The PACE4 zymogen loses its signal peptide within the ER, via the signal peptidase. It is also N-glycosylated at various sites at this point. The prosegment

at the N-terminus also acts as a chaperone and an autoinhibitor after it is cleaved and remains associated with the remaining portion of the protein. This precursor is folded into an inactive conformation within the lumen of the ER. As with furin, the prosegment causes PACE4 to remain inactive. Unlike furin, PACE4 is a secreted protein that works primarily on the cell surface. Complete activation of PACE4 can occur at two locations. The first location is in the trans-Golgi network. Second, PACE4 can be fully activated at the cell surface via C-terminal Cys-rich domains binding to the surface of the cell (Nour et al., 2005). At the cell surface, PACE4 can bind heparin via its heparin-binding region located between residues 743 and 760. The heparan sulfate proteoglycan-PACE4 complex allows PACE4 to be anchored to the cell surface. (Tsuji et al., 2003).

Aside from similar trafficking patterns, PACE4 and furin share similarities in function and structure. PACE4 and furin have two conserved disulfide bonds that are present in the catalytic domain. The bonds in PACE4 are located at C355 – C385 and C263 – C412, while in furin they are found at C303 – C333 and C211 – C260 (Henrich et al., 2003). Structurally, both proteins are similar. The signal peptide, propeptide, catalytic domain, P domain, and cysteine-rich domain are all shared by both proteins. The only difference between the two is that PACE4 lacks a transmembrane domain and a cytoplasmic tail due to it being a secreted protein. There is overlap with function and the substrates that these two proteins cleave. As mentioned previously, PACE4 and furin both cleave protein precursors at certain single or paired basic amino acid sequences that are inside the same motif (Arg-X-X-Arg), which leads to redundancy in substrates. However, furin is expressed in more cell types compared to PACE4, leading to broader substrate pool (Seidah and Prat, 2012). Since PACE4 and furin are similar,

we expect to see correlations between the trafficking and function of PACE4 and furin when each disulfide bond is removed.

In our lab, the formation of C211 – C260 and C303 – C333 bonds in furin were inhibited by changing each of the cysteine residues into serine to examine the significance these bonds have on the protein's trafficking and function (Eckwahl et al., in preparation). To examine changes in function, pro-von Willebrand's factor (pro-vWF) was expressed with the mutant forms of furin. Pro-vWF is easily processed by wild type furin. Altering any of the four cysteine residues prevented furin proteins from processing pro-vWF, indicating that both disulfide bonds must be intact for proper furin activity. The lab previously showed that the C303 – C333 bond is needed for propeptide excision and furin trafficking beyond the ER. Furin mutants unable to form this bond could not excise the propeptide and were retained in the ER. The propeptide mediates the first folding event in the ER, which is necessary for transit into the ERGIC. It was proposed that C303 – C333 is needed for the structural integrity of two Beta loops that take part in binding a calcium ion. This calcium ion could stabilize one of the furin substrate-binding pockets.

In contrast, C211S and C260S mutants could leave the ER and travel to the Golgi. While furin leaving the ER indicates that these two mutants could fold into a conformation that was similar enough to wild type furin that they could exit the ER, they still did not have the specific secondary folding that would allow for complete catalytic activity against pro-vWF. C211-C260 is close to the catalytic triad and could take part in holding the correct positioning of amino acids in the catalytic triad, which could explain why catalytic activity on pro-vWF from the C211S and C260S furin mutants was

abolished. Interestingly, propeptide autocatalytic cleavage was still carried out by the C211S and C360S mutants, but our results suggest the acidic environment of the Golgi induces this cleavage at R107 (Eckwahl et al., in preparation). However, these results conflict with the model for furin maturation that is currently known, which states that cleavage of the propeptide at R107 is needed for trafficking beyond the ER. The trafficking and modifications of the C211S and C2360S mutants have been thoroughly tested, but, because of the controversial nature of these results, further information needs to be shown to confirm our findings about furin. The current study aimed to examine the importance of each disulfide bond in PACE4, a PC similar to furin.

We will examine the effect these bridges have on PACE4 trafficking and function by inhibiting the ability of the protein to form these bonds. It is expected that the results from this study will be similar to the results from the furin study. This could mean that both bridges in PACE4 will be needed for catalytic activity. We would also predict that C355 – C385 will be needed for propeptide excision and PACE4 trafficking beyond the ER. Most importantly, we would expect C263 – C412 will be necessary for full catalytic activity but not propeptide excision. This will also indicate if propeptide excision can occur in the Golgi, contradictory to what is currently thought. This project will allow us to not only gather information on the importance of each disulfide bond in PACE4, but also allow us to further back up our claims about furin trafficking and function that we discovered in our previous study.

Materials & Methods

PACE4 Expression Plasmid: *E. coli* cells harboring a construct containing PACE4-pSVL plasmid were inoculated in LB broth containing 200 µg/mL ampicillin (LBA) and incubated for 24 hours at 37 Celsius. Plasmid DNA was purified with a QIAprep mini kit (Qiagen; Valencia, CA). Using *XhoI* and *BamHI*, a restriction digest of pcDNA3.1 and PACE4-pSVL was performed, followed by an agarose gel to resolve the digested DNA. The pcDNA3.1 and PACE4 DNA bands were excised under long wave UV light. A Wizard PCR Preps DNA purification system (Promega; Madison, WI) was used to purify the DNA. To quantify purified pcDNA3.1 and PACE4 cDNA, an analytical gel was performed. T4 ligase (Invitrogen; Carlsbad, CA) was used to ligate the pcDNA3.1 and PACE4 cDNA together, creating a unique PACE4 expression plasmid. This plasmid was transformed into *E. coli* XL1-Blue cells using the calcium chloride technique. These cells were plated on LBA agar at 37 Celsius for 24 hours. The pcDNA3.1 – PACE4 plasmid was purified using another QIAprep spin miniprep kit.

Site-Directed Mutagenesis: We used GenBank to carry out a multiple sequence alignment to determine the locations of cysteines that form the disulfide bonds in human PACE4 and human furin, which were found to be located at C355/C385 and C263/C412. The GeneArt Site-Directed Mutagenesis System (Invitrogen) was used to carry out the site-directed mutagenesis reaction. Primers were designed according to the mutagenesis protocol provided in the kit and were produced by Invitrogen. Mutated DNA was introduced into One Shot® MAX Efficiency® DH5α™-T1R competent cells that were provided in the mutagenesis kit via the calcium chloride method. The mutated plasmid was recovered by using a Qiaprep mini kit and a restriction digest confirmed the

presence of PACE4 plasmid DNA. Mutated plasmid DNA was sequenced at the DNA Sequencing Core facility located at the University of Michigan (Ann Arbor, MI) to confirm that the mutation was present.

Preparation of Co-expression Assay: Using a 35 mm-6 well cell culture plate, 50,000 RPE.40 cells were seeded to each plate and incubated for 20 hours in 3 mL DMEM/F12 (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals; Flowery Branch, GA). 7.5 uL TransIT reagent (Mirus; Madison, WI) in 250 uL serum-free DMEM/F12 was added into each well. 2.5 ug plasmid DNA (wild type PACE4/pro-vWF, C355S PACE4/pro-vWF, and pro-vWF) was then added into the corresponding wells. After roughly 4 hours, the original DMEM/F12 medium was removed and each well was washed three times with phosphate-buffered saline (PBS). Another 3 mL of serum-free DMEM-F12 was added into each well and then the cells were incubated for 16 hours. Conditioned medium was collected and concentrated from 2 mL to 20 µL using Amicon Ultra 2mL 30K centrifugal filters (Millipore; Cleveland, OH). Conditioned medium was frozen after concentration.

Analysis of Co-expression Assay: Using the Bolt Western Blot system (Invitrogen), concentrated conditioned medium was resolved through SDS-PAGE with a 4%-12% gradient gel. Following this, the proteins on the gel were transferred to an Immobilon-P membrane (MilliporeSigma; Temecula, CA). The membrane was blocked with 2% milk in PBS and incubated overnight with anti-von Willebrand's factor antibody (Dako; Santa Clara, CA) that was diluted 1:1000. Following 3 washes in 0.6% milk in PBS, the membranes were incubated with anti-rabbit IgG alkaline phosphatase conjugate (Promega; Madison, WI) for 1.5 hours. This secondary antibody was diluted

1:4000. The membrane was washed 2 more times with 0.6% milk in PBS and 1 time with Western Blot Substrate Buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 5 mM MgCl2). Nitro blue tetrazolium and 5-bromo-4 chloro-3-indolyl-1-phosphate (Promega) were added to start color development. Results were quantified by comparing band intensities using ImageJ software.

Activity Assay: A mixture of 20 uL conditioned medium, 5 uL [10mM] R-T-K-R synthetic substrate (Peptides International; Louisville, KY), and 20 uL of either PBS or serum-free medium were added into wells on a 96-well plate. Conditioned medium was handled in the same way as the conditioned medium used in the co-expression assay. After covering the wells with parafilm to diminish evaporation, the plate was placed in a 37 Celsius incubator for 1 hour. The plate was then placed in a Synergy HTX multi-mode reader (BioTek; Winooski, VT) with an excitation wavelength of 360 nm and a emission wavelength of 460 nm in order to quantify the fluorescence of each well. Background fluorescence was subtracted from both samples.

Results

Site-Directed Mutagenesis:

Due to time constraints and numerous setbacks, we were only able to generate one mutation out of four possible mutations: the C355S. The triplet codon for C355 was changed from TGC (specifying cysteine) to TCC (specifying serine), which was confirmed through sequencing of the mutant PACE4 plasmid. This effectively removed the ability of PACE4 to form a disulfide bond between 355 and 385.

Co-expression Assay:

We determined that there was a distinct difference in the amount of pro-vWF that was processed between the conditioned medium that was transfected with wild type PACE4 and pro-vWF compared to the conditioned medium that was transfected with the C355S PACE4 mutant and pro-vWF. The negative control, which was only transfected with pro-vWF, showed two bands located at roughly 350 kDa and 250 kDa. These two bands represent pro-vWF and processed vWF, respectively (Figure 1A). This indicates that there is something unknown in the medium that is processing some of the vWF present in the medium. In the WT lane, nearly all vWF that was present in the medium was processed, indicated by the single band located at around 250 kDa and the absence of the larger 250 kDa band (Figure 1A). Lastly, the C355S mutant lane is identical to the control lane with two bands located at 350 kDa and 250 kDa (Figure 1A). At first, this may seem that there is some amount of processing being carried out with the C355S mutant. However, due to the similarity in the negative control and C355S lane, we can suggest that there is no processing in the C355S lane (Figure 1A).

Furthermore, through the quantitative analysis of the western blot these results are clearer and more apparent. WT PACE4 processed about 3.5 times more pro-vWF when compared to the C355S mutant (Figure 2B).

Figure 1A

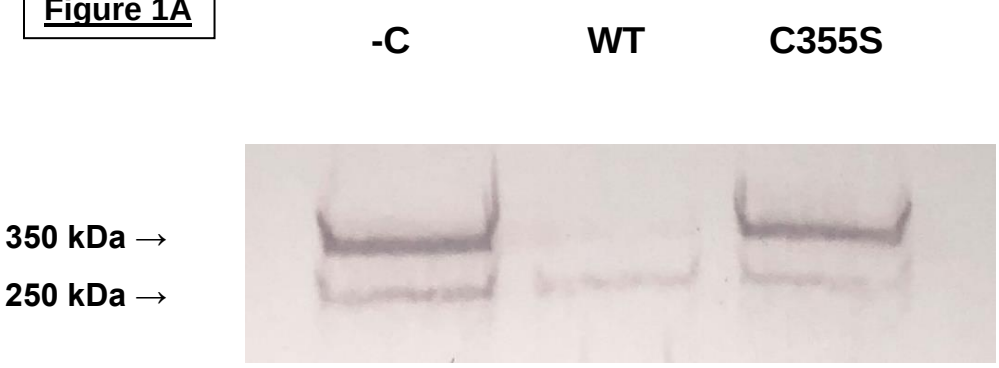

Figure 1B

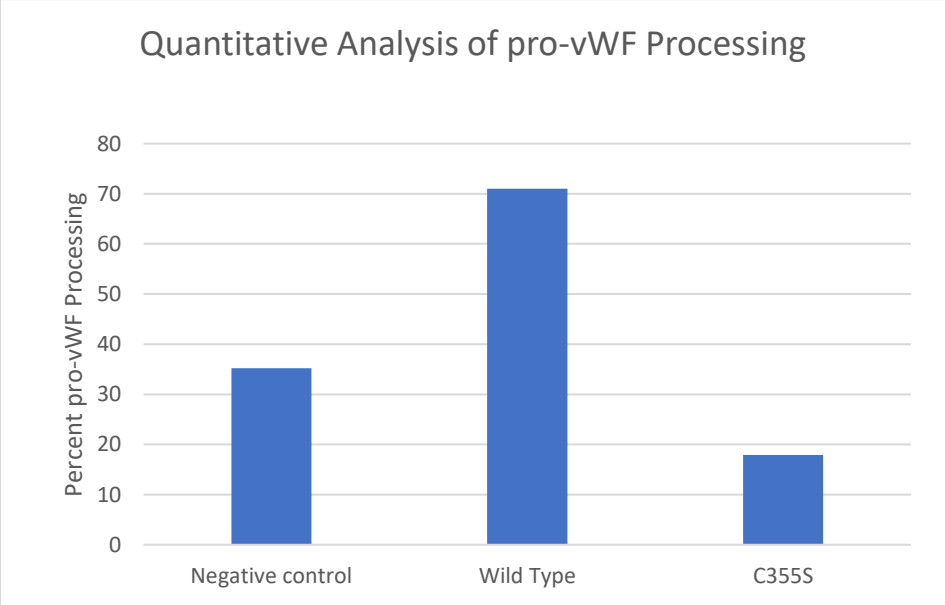

Figure 1: A western blot analyzing the co-expression experiment by examining processed vWF and unprocessed vWF. RPE.40 cells were transfected with pro-vWF/WT PACE4, pro-vWF/C385S PACE4, and pro-vWF. Samples of concentrated medium were subjected to western blotting using an antibody to vWF. Pro-vWF is 350 kDa, while mature, processed vWF is 250 kDa. Figure 1A shows the western blot, while Figure 1B represents quantitaitive analysis of the percentage of pro-vWF processing for the negative control, WT, and C355S, done using ImageJ.

Activity Assay:

Conditioned medium was also used to determine PACE4 activity using a synthetic peptide (R-T-K-R) conjugated to a fluorescent dye; cleavage of the peptide by PACE4 liberates the dye, allowing for fluorescence to be measured. There was 13% more activity observed when wild-type PACE4 was expressed than when the C355S mutant was expressed (Figure 2), which is consistent with the Western blot results.

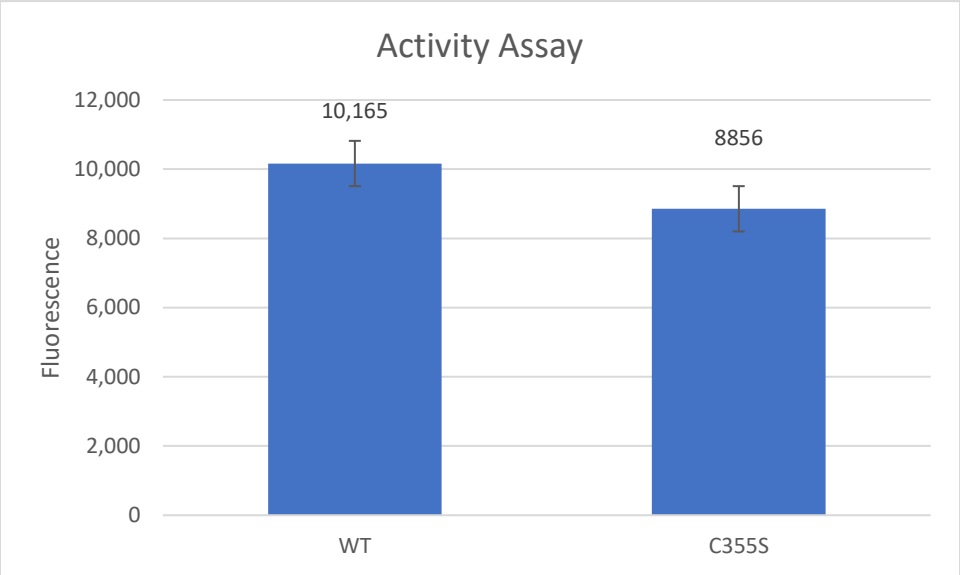

Figure 2: Activity assay examining conditioned medium from RPE.40 cells expressing wild type or C355S PACE4. Conditioned medium was combined with a synthetic peptide conjugated to a fluorescent dye and was incubated at 37 Celsius for 1 hour. Fluorescence was measured on a plate reader and background was subtracted from both samples. Error bars were calculated by using standard error.

Discussion

C355 in PACE4 plays a key role in the function of PACE4. This is shown through the co-expression assay and activity assay (Figure 1 and Figure 2). The C355S PACE4 mutant showed a lower amount of pro-vWF processing when compared to wild type PACE4 in both assays. This indicates that the C355-C385 disulfide bond plays a crucial role in the ability of PACE4 to fully process substrates such as pro-vWF and corroborates the role of the C303-C333 bond in furin. The C303-C333 disulfide bond in furin is needed to keep the structural integrity of the two beta loops stable which take part in binding a calcium ion. In PACE4, the C355-C385 disulfide bond is homologous to the C303-C333 disulfide bond in furin. Therefore, we suspect that the C355-C385 bond is responsible for also stabilizing these two Beta loops in PACE4 and binding a calcium ion. This would also be consistent with the strict calcium dependence of PACE4 (Sucic et al., 1999).

While we currently have no direct trafficking data available for the C385S PACE4 mutant, we can suggest that trafficking has been hindered. Trafficking and function of a protein are directly linked. A protein will be unable to carry out its proper physiological functions if it is not trafficked to the correct location. PACE4 may not correctly function if it is not secreted from the cell. Since the activity of C385S PACE4 was diminished, we can hypothesize that C385S PACE4 was unable to be secreted from the cell. Therefore, the 355-385 disulfide bond may play a role in the maturation process of PACE4. Elimination of C303-C333 in furin prevents trafficking beyond the ER, presumably due to the inability to fold properly. It seems that the same is happening when C355-C385 is eliminated in PACE4. As in furin, the propeptide in PACE4 is necessary for the first

folding event that occurs during the maturation of PACE4, which is needed for the protein to be trafficked to the ERGIC. Lack of the C355-C385 bond could interfere with the proper spatial positioning of the propeptide, and thus inhibit its chaperone function. We can also speculate that autocatalytic propeptide cleavage is inhibited, which occurs when the C303-C333 bond is eliminated in furin. Our two assays are consistent with these findings as well. Both assays showed a reduction of processed pro-vWF in C355S mutant indicating that either this mutant was able to be secreted from the cell or it was retained in the ER. The more likely outcome was this mutant was retained within the ER, since this is what occurs in the homologous mutant in furin. If the mutant version of PACE4 is retained in the ER, the processing of the synthetic substrate would likely be the result of some endogenous protease that the cells are secreting. However, further testing must be done to elucidated what is happening here. Specially, a Western blot on conditioned medium looking PACE4 should be performed.

There were an excessive number of setbacks throughout this study, which is why we were only able to generate one mutant and do assays that were directly related to activity. Most of the setbacks were during site-directed mutagenesis. We exhausted what seemed to be every possible variable in an attempt to get more than one mutant. Before switching to the GeneArt site-directed mutagenesis kit, we used another kit called QuikChange II (Invitrogen). The first and simplest variables that we changed while using this kit were the amounts of template DNA, primer DNA, and DNA polymerase that were added to each reaction. Changing these variables still gave no successful mutagenesis. A restriction digest on the PCR product was done to check if a sufficient amount of DNA was present in order to efficiently carry out the transformation

step of site directed mutagenesis (See appendix). The intensity of the bands shows that there is PACE4 DNA, indicating that this was not the issue. After repeating the mutagenesis reaction multiple times, we switched to the GeneArt kit. The second reaction with the new mutagenesis kit generated the C355S mutant. However, we failed to generate any of the other mutants even after going through all the same variable changes that were done with the QuikChange II kit. Another major issue that we encountered was getting proteins expressed in RPE.40 cells. Before this study, we had only been able to express pro-vWF in RPE.40 cells while using a specific cell medium that was discontinued in 2012. The first course of action to fix this issue was to try multiple different cell media that were specific to CHO cell lines, since RPE.40 cells are a CHO cell line. None of these cell media were useful for pro-vWF expression. After multiple failed attempts, we supplemented DMEM/F12 medium with ITS (insulin, transferrin, and selenium; Invitrogen). This resulted in a drastic change in expression and clarity of bands present on blots. We then were able to gather activity data through the co-expression assays.

In conclusion, the goal of this study was not only to generate data on the roles of each disulfide bond in PACE4 trafficking and function, but to also compare these disulfide bonds to their counterparts in furin. According to our preliminary data on furin, removing either of the disulfide bonds in furin abolished the ability of furin to process pro-vWF. The homologous bond to C303-C333 is the C355-C385 disulfide bond in PACE4. We demonstrated that through the removal of the C355-C385 disulfide bond in PACE4 by mutating the 355 cysteine inhibited the ability of PACE4 to process pro-vWF. This indicates that this specific disulfide bond is critical for retaining the ability of PACE4

to cleave substrates. Additionally, we can make an educated guess as to where PACE4 is trafficked to within the cell even with no direct trafficking data. PACE4 was most likely retained in the ER since no pro-vWF was processed. These results are similar to our results pertaining to the relationship between furin and its disulfide bonds, which is what we expected to see.

Our future study plans can be divided into three phases. The first phase would be to generate the remaining PACE4 mutants and finish examining the functional repercussions brought by removing the C263-C412 disulfide bond. The second phase would be to generate trafficking data on the 4 PACE4 mutants. Lastly, phase 3 would involve examining the importance of the disulfide bonds in other PCs.

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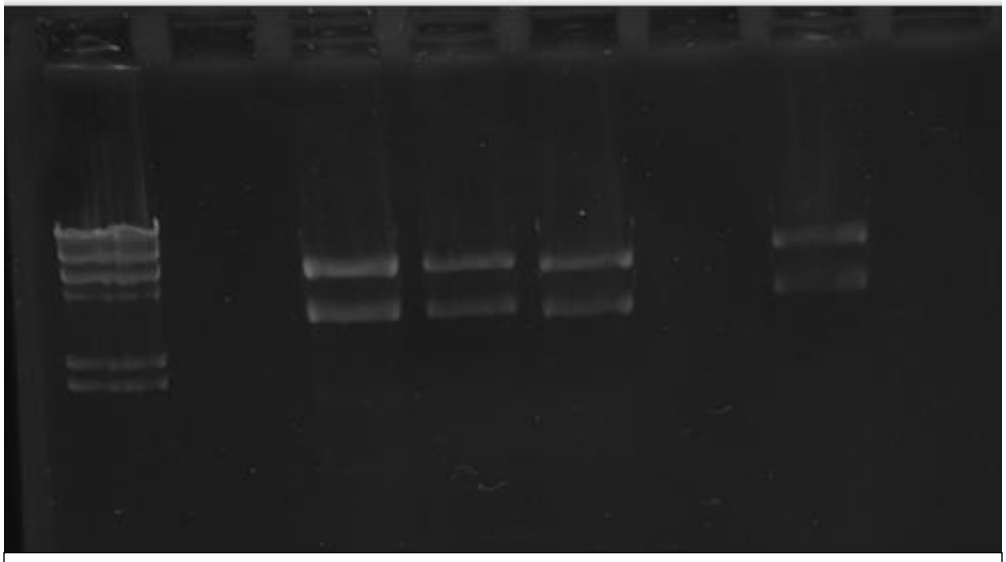
Appendix: Restriction Digest



Appendix 1: Restriction digest of site-directed mutagenesis PCR product to examine if a proper amount of PACE4 DNA was present. All lanes except for the molecular weight standard represent different samples of wild type PACE4.